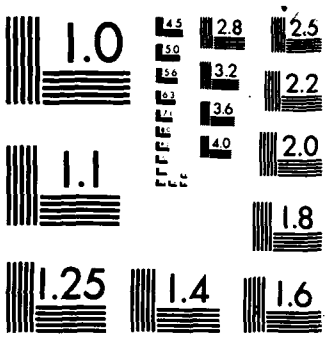
AD-A141 629 ACTIONS OF INTERFERONS ON MACROPHAGES(U) TRUDEAU INST 1/1
INC SARANAC LAKE NY E A HAYELL 01 JUN 84
N00014-83-C-0407

UNCLASSIFIED F/G 6/5 NL



SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: 1	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): Actions of Interferons on Macrophages	5. TYPE OF REPORT & PERIOD COVERED: Annual Report 6/1/83-5/31/84
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Edward A. Havell, Ph.D.	8. CONTRACT OR GRANT NUMBER(s): N00014-83-C-0407
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Trudeau Institute, Inc., P.O. Box 59, Saranac Lake, NY 12983	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: Jeannine A. Majde, Ph.D., Scientific Officer, Immunology Code 441, Cellular Biosystems Group, Dept. of the Navy, ONR Arlington, VA 22217	12. REPORT DATE: 6/1/84
	13. NUMBER OF PAGES: 9
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Unlimited

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Murine interferon gamma (MuIFNγ), monoclonal anti-MuIFNγ, antiviral activities, macrophage activation, macrophage activation factor (MAF), neutralization, immunization, purification.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In addition to antiviral activity, the three antigenically distinct species (α,β,γ) of interferon (IFN) exhibit multiple biological activities, some of which suggest, possible immunomodulatory roles for these molecules in either the generation and/or expression of immunity. The ultimate objective of our research is to determine what actions each IFN may mediate during cell-mediated immune responses. Our immediate goal is to study the effects of these antiviral molecules on macrophage functions, both in vivo and in vitro murine systems. Interest in IFN actions on macrophage function(s) is based on the

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

S/N 0102-LF-014-6601

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

facts that this cell is an intergral element in cellular immunity, often executing affector functions, as well as the reports that various preparations possessing antiviral activity mediated by IFN enhanced the expression of many parameters associated with activated macrophages. To establishe whether a particular effect is due to an IFN, either pure murine IFNs (MuIFN) or their respective monospecific or monoclonal neutralizing antibodies are required. Our efforts over the past year have centered on the characterization of the lymphokine MuIFNγ and the generation of neutralizing monoclonal anti-MuIFNγ which is being used as a probe in vitro and eventually will be used in vivo, to elucidate MuIFNγ actions on macrophages and the role of this T-cell product in cellular immunity. The following is a list of our accomplishments.

1. The successful immunization of rats with a partially purified IFNγ produced by the closely related murine species.

2. The generation of transspecies hybridomas through the fusion of rat immune B-cells to mouse myeloma (P3U-1) cells. Screening the supernatants of hundreds of wells containing hybridomas from multiple fusion efforts, revealed that the medium from a single well possessed the ability to neutralize the antiviral activity of MuIFNγ. From this well, a hybridoma secreting a rat anti-MuIFNγ monoclonal antibody (MAb) was cloned.

3. The hybridoma producing rat anti-MuIFNγ MAb has proven stable and is being propagated both in vitro and as well as in vivo in nude mice as ascites. One ml of crude culture supernatant can neutralize 2000 units of MuIFNγ antiviral activity, whereas, the ascitic fluids are 1,000 times more potent.

4. Characterization studies as to the potential of the MAb to neutralize various biological activities of lymphokine preparations thought to be mediated by MuIFNγ, have been initiated. These studies have so far established that the MAb neutralizes not only the antiviral and anticellular (growth inhibition) activities of crude lymohokine preparations but also, the ability of these preparations to activate the tumoricidal action of macrophages. This latter observation indicates that MuIFNγ is also macrophage activation factor (MAF).

Generation of Rat anti-MuIFNγ Monoclonal Antibody (MAb) and Its Neutralizing Activities

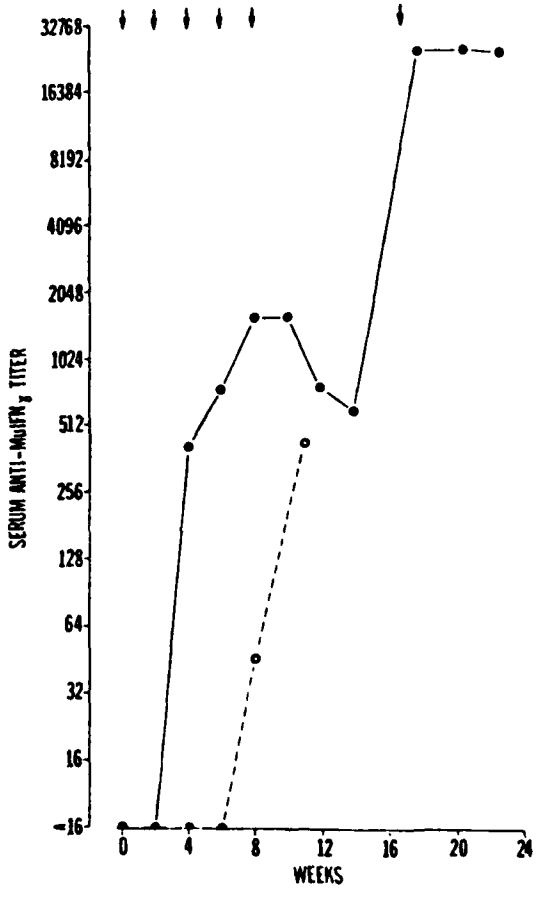
1. Immunization of rats with MuIFNγ. High-titered preparations (1000 units/ml) of MuIFNγ were produced in vitro by stimulating spleen cells (10^7 cells/ml), obtained from $AB6F_1$ mice challenged intravenously 6 days earlier with an immunizing Listeria dose ($4x10^3$ organisms), with the polyclonal T-cell mitogen phytohemagglutinin (PHA). The 24 hr culture supernatants were concentrated 10-fold by Amicon PM-10 ultrafiltration. A MuIFNγ fraction (60% total activity) was isolated, purified, and further concentrated (60,000 units/ml) by means of concanavalin A (Con-A) lectin affinity chromatography. This major fraction of MuIFNγ which bound to the lectin and was specifically eluted with the counterligand α-methyl-D-mannoside was purified 50-fold over the starting preparation to a specific activity of $5x10^5$ units/mg of protein. This partially purified Con A-bound MuIFNγ served as the immunogen in the following studies.

Initially, rats (DA strain) were injected with a total of 2 ml of MuIFNγ-adjuvant emulsion at multiple subcutaneous sites on a biweekly basis. The first two biweekly immunizations were done with Freund's complete adjuvant, which was emulsified with an equal volume of the Con A-bound MuIFNγ (32,000 units/ml). All subsequent immunizations were carried out with the same MuIFNγ preparation emulsified with Freund's incomplete adjuvant. Prior to each immunization, animals were bled, sera collected and heat-inactivated for 30 min. Sera were stored at -20°C, until assayed for anti-MuIFNγ neutralizing activity. The anti-MuIFNγ neutralizing titer of an antibody preparation is defined as the reciprocal of the highest dilution of the antibody which when reacted with an equal volume of MuIFNγ (final concentration of 10 antiviral units/ml) neutralizes 50% of the antiviral activity as judged by the development of vesicular stomatitis virus cytopathic effect on L929B cells.

The appearance of titers of serum neutralizing anti-MuIFNγ antibodies during the course of immunization of both a typical rat as well as a rabbit are presented in Fig. 1. While both the rat and rabbit were immunized according to the same schedule with the same doses of antigen, the rat responded with neutralizing anti-MuIFNγ later and with lower levels of neutralizing activity than the rabbit. The specificities of both polyclonal anti-MuIFNγ neutralizing sera were examined in neutralization assays against a variety of IFN preparations. Neither anti-MuIFNγ sera neutralized the antiviral activities of MuIFNα/β or HuIFNγ preparations. The rabbit and rat anti-MuIFNγ sera, which were raised against the Con A-bound MuIFNγ, neutralized the total antiviral activity of the crude unfractionated MuIFNγ from which the immunogen was isolated. This indicates that these antisera also neutralize the antiviral activity of the MuIFNγ component present in the unfractionated preparation, which does not bind to immobilized Con A (Con A unbound MuIFNγ). The rat anti-MuIFNγ did not cross-react to neutralize rat IFNγ whereas, the rabbit anti-MuIFNγ did.

Fig. 1

Immunization Schedules and Appearance of Rabbit (●) and Rat (○) serum Anti-MuIFNγ Neutralizing Activities. Time of Immunization (↓).



This indicates that the rabbit recognized epitopes common to both murine and rat MuIFNγ and based on the specificity of the rat immune serum, it can be concluded that the rat recognizes an antigenic determinant(s) unique to the MuIFNγ molecule.

2. Derivation of a Hybridoma secreting Rat anti-MuIFNγ Monoclonal Antibody. The successful immunization of rats with MuIFNγ enabled us to fuse rat immune spleen B cells with mouse myeloma cells (P3U-1) in an attempt to produce a hybridoma which secretes rat neutralizing anti-MuIFNγ antibody. The following is a brief description of the procedures used to obtain a hybridoma producing monoclonal

anti-MuIFNγ. Once rats were found to possess high-titered serum anti-MuIFNγ activity, they were again immunized intravenously. Four days later, the rat spleen cells were mixed at a 5:1 ratio with mouse P3U-1 myeloma and hybridomas were generated in the presence of polyethylene glycol 1450. Cells were suspended in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum (FBS) and seeded into 16mm wells. The following day, all wells received an additional equal volume of medium containing 2x hypoxanthine, aminoterin and thymidine (H.A.T.). This medium was removed one week later and the cultures replenished with medium containing only H.T. After 7 to 10 days, the hybridoma supernatants were screened for antibodies capable of neutralizing the antiviral activity of MuIFNγ. Following three fusion and screening efforts, the supernatant of a single well from hundreds of wells containing the fusion products from the fourth immune rat possessed neutralizing activity for MuIFNγ. Positive hybridomas from this well were subcloned twice by limiting dilution in microtiter plates with irradiated thymocyte feeder layers in DMEM containing 20% FBS. The cloned hybridoma (R4-6A2) was expanded and the isotype of monoclonal antibody (MAb) produced by this clone was determined to be in the rat IgG_1 subclass. Culture supernatants were made to 45% saturation and the precipitate (IgG) was resuspended in phosphate buffered saline (PBS) pH7.4 to 1/30 original volume, dialyzed exhaustively in PBS and then sterile filtered.

The specificity of the neutralizing activity of the concentrated MAb was tested against a variety of MuIFNγ preparations. The results in Table 1 reveal that irregardless of the source of MuIFNγ, the antiviral activity of each MuIFNγ preparation was neutralized to an approximately similar degree as the immunogen (PHA-induced MuIFNγ, Con A-bound fraction). In contrast, the antiviral activities of MuIFNα+MuIFNβ preparations, human IFNγ and rat IFNγ were not neutralized by the MAb. The observation that the antiviral activities of glycosylated and non-glycosylated (recombinant MuIFNγ produced by E. coli) MuIFNγ were neutralized equally, indicates that the epitope recognized by the MAb resides within the polypeptide structure of the MuIFNγ molecule.

Further characterization studies of the MAb has established that the amount of MAb required to neutralize MuIFNγ is directly proportional to the titer of antiviral activity (antigenic mass). In Figure 2, a dose-response curve for the MAb neutralization of homologous (murine) and heterologous (rat) antiviral activities reveals a linear relationship exists between the quantity of MAb required to neutralize a defined amount of antiviral activity (antigenic mass). Since the rat fibroblast cells are 2-times less sensitive than homologous murine cells to MuIFNγ antiviral activity, twice as much MAb was required to abate an equivalent amount of MuIFNγ antiviral activity on rat cells than a similar amount of antiviral activity on homologous murine cells.

TABLE 1

Specificity of Rat Monoclonal Anti-MuIFNγ

Interferon	Production Mode: System	Production Mode: Induction	Fraction	Neutralization titer[a] for antiviral activity
MuIFNγ	Spleen cells	Con-A[b]	-	14,000
MuIFNγ	Spleen cells	PHA[c]	-	13,000
MuIFNγ	Spleen cells	PHA[d]	Con-A-Bound[d]	13,000
MuIFNγ	Spleen cells	PHA[d]	Con-A-Unbound[d]	13,000
MuIFNγ	recombinant DNA[e]	-	-	13,000
MuIFNγ	Serum	BCG/OT[f]	-	13,000
MuIFNγ	MLR	Allogeneic cells[g]	-	26,000
MuIFN α+β	C-243	virus	-	<20
Rat IFNγ	Spleen cells	PHA[i]	-	<20
HuIFNγ	Blood leucocytes	PHA[j]	-	<20

Neutralization titer is defined as the reciprocal of the highest dilution of antibody which when mixed with an equal volume of IFNγ (Final IFNγ concentration 10 U/ml) neutralizes 50% of the antiviral activity as judged by the development of viral cytopathic effect.

Spleen cells from AxC57BL/6F$_1$ mice injected 6 days earlier with 2X10^3 Listeria monocytogenes were suspended at 10^7 cells/ml in RPMI 1640 with 10μg/ml gentamycin (Schering) and stimulated in vitro with 2μg/ml Con-A (Type IV; Sigma) for 24 hrs.

Same as in b, except spleen cells were stimulated with 5μg/ml PHA (Burroughs-Wellcome).

A PHA-induced MuIFNγ was fractionated by Con-A affinity chromatography into two fractions: one fraction which specifically bound to the lectin column (Con-A-bound) and a minor fraction which did not bind (Con-A-unbound).

Highly purified IFNγ (>10^{7}U/mg) produced by cloned MuIFNγ gene transfected to E. coli and kindly supplied by Genentech, Inc.

AxC57BL/6F$_1$ mice were injected with 5x10^6 Mycobacterium bovis (BCG) and 21 days later inoculated with old tuberculin (O.T.;10 mg total protein; Jensen-Salisbury Labs). Serum was collected 2 hrs. after injection of old tuberculin.

For mixed-lymphocyte reaction (MLR)-induced IFNγ, responder C57BL/6xDBA/2 F$_1$ mice were injected in both hind footpads 9-21 days earlier with 2x10^6 allogeneic (H-2^a) SA-1 tumor cells. Responder spleen cells were mixed with an equal number (4-5x10^6) of gamma-irradiated (1000r) stimulator spleen cells from A strain mice and superanants were collected 72 hours later.

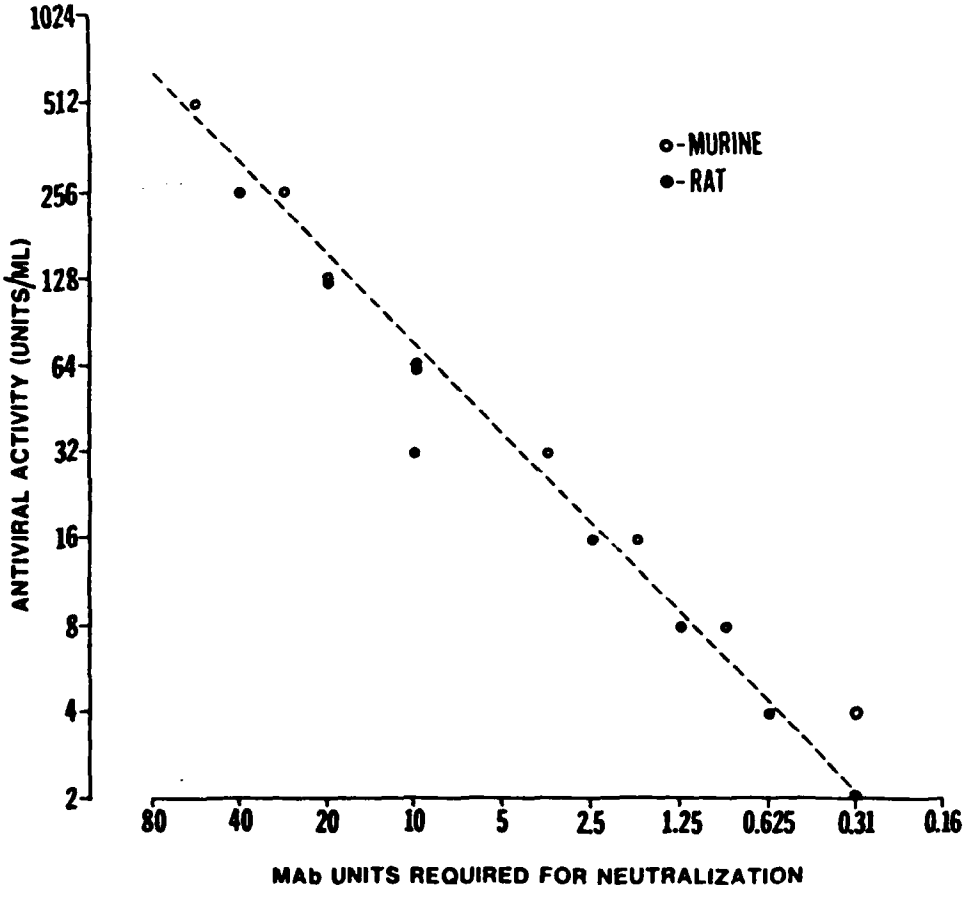
Pure IFNα+β was produced by Newcastle disease virus-induced mouse C-243 cells and purchased from Enzo Biochem.

Spleen cells from DA rats injected 6 days earlier with 5x10^6 Listeria monocytogenes subcutaneously, were suspended at 10^7 cells/ml in RPMI 1640 with 10μg/ml gentamycin (Schering) and stimulated with 5ug/ml PHA (Burroughs-Wellcome) for 24hrs.

Human IFNγ induced by PHA induction of blood leucocytes and generously supplied by Flow Laboratories, Inc.

Fig. 2

Relationship Between Amount of MAb Required to Neutralize Different Concentrations of MuIFN$_\gamma$ Antiviral Activity on Murine and Rat cells.



3. Production and Purification of Rat anti-MuIFN$_\gamma$ MAb. The rat-anti MuIFN$_\gamma$ MAb is currently being used to determine what effects MuIFN$_\gamma$ has on macrophages and eventually will be used in vivo to establish what role(s), if any, this lymphokine may mediate in the generation and/or expression of cell-mediated immunity. To achieve these goals, large quantities of high-titered purified MAb will be required. Fortunately, the transspecies hybridoma (R4-6A2) has proven stable and is being propagated both in culture and as ascites in nude (nu/nu) mice. The

average neutralizing titer/ml of ascitic fluids is $2x10^5$ neutralizing units against 10 MuIFNγ antiviral units, which is 1000-times more potent than that obtained in vitro under optimal culture conditions. Through means of radial immunodiffusion, it has been established that approximately 25% of the total ascitic fluid protein (60mg/ml) content is rat IgG_1 protein.

A number of different methods have been used to attempt the purification of the rat IgG. These have included $(NH_4)_2SO4$ precipitation, Protein-A affinity chromatography, as well as antibody affinity chromatography. Anion exchange chromatography using DEAE-Biogel has proven very satisfactory due both to the tremendous binding capacity of the exchanger for protein (45mg/1ml beads) and the mild conditions used to elute the MAb from the column. Based on theoretical considerations, it is currently believed, that this procedure may result in the total purification of IgG. Current studies are underway to determine if the rat MAb has been purified to homogeneity, for if this has been achieved, it will allow us to use commercially available rat IgG protein of similar protein content to that of the MAb for a mock preparation in future in vivo studies.

4. The effect of MAb on the macrophage activation potential of lymphokine preparations. Evidence indicates that IFNγ can activate macrophage tumoricidal activity. One unresolved question is whether this is the only lymphokine affecting macrophage tumor cytotoxicity. To establish if MuIFNγ is responsible for this activity, several experimental procedures were performed to determine whether the rat-anti MuIFNγ MAb could eliminate the ability of crude lymphokine preparations to activate macrophages to destroy tumor cells in vitro.

a. Neutralization Studies. To test whether the rat anti-MuIFNγ MAb possessed the ability to neutralize the macrophage tumoricidal activity of a different lymphokine preparations, an in vitro macrophage tumoricidal assay based on the one originally described by Pace and Russell was developed. Macrophages were harvested from the peritoneal cavities of $AB6F_1$ mice injected 3 days earlier with 1.0 ml of a 10% solution of Proteose-peptone. These cells were washed and resuspended at 10^6 cells/ml in RPMI-1640 medium supplemented with sodium pyruvate and 5% FCS. To wells of a microtiter plate 0.2 ml of cells were allowed to adhere. Two hrs later, non-adherent cells were washed off and 0.2 ml of test lymphokine preparations were added to groups of triplicate wells. Prior to their addition to the macrophage cultures, the test lymphokine preparations were reacted with and without varying concentrations of the MAb for 1 hr and then incubated with the macrophages in the presence of 10ng of endotoxin. Five hrs later, ^{51}Cr labeled tumor target cells (P815 mastocytoma) were added to each well at an effector to target ratio of 5:1. The uppermost 0.1ml of fluid from each well was collected 16 hrs later and the tumoricidal activity was measured as % specific ^{51}Cr release. It should be mentioned that the quantity of MuIFNγ used in these macrophage activation studies was the lowest amount of antiviral activity (units/ml) which consistently induced the maximum expression of tumor cytotoxicity.

The results of these studies are presented in Table 2, were it is evident that crude, partially purified (Con A-bound) and highly purified (recombinant : 10^7U/mg) MuIFNγ preparations all elicited macrophage tumoricidal activity. In contrast, a MuIFNα/β preparation induced only marginal activity when incubated at a 10-fold higher concentration than that required for MuIFNγ preparations to induce maximum cytolysis. When test lymphokines were preincubated for 1 hr with twice the amount of MAb required to neutralize 50% of the stated MuIFNγ concentrations, the antiviral activity was abolished, while the macrophage tumoricidal activity was

TABLE 2

Inhibition of Lymphokine-induced Macrophage Tumoricidal Activity by MAb to MuIFN

IFN Preparation	Relative excess of MAb[a]				IFN titer[h] (Units/ml)		
	No MAb	2x	5x	10x	No MAb	+excess MAb 2x	5x
	% Specific ^{51}Cr Release[g]						
Con-A-induced MuIFN γ[b]	30.8±1.4	21.4±3.4	14.2±0.5	10.5±1.2	8	<4	<4
PHA-induced MuIFN γ[c]	31.3±1.4	23.0±1.4	13.8±1.0	11.1±2.3	8	<4	<4
PHA-induced MuIFN γ[d] (Con-A-Bound Fraction)	29.1±3.3	17.5±2.9	11.3±2.3	10.5±1.7	16	<4	<4
PHA-induced MuIFN γ[d] (Con-A-Bound Fraction)	33.4±2.8	26.7±1.2	20.0±3.2	13.6±3.1	8	<4	<4
recombinant DNA MuIFN$_\gamma$[e]	32.6±2.0	23.8±1.8	14.7±2.1	10.6±1.7	16	<4	<4
MuIFN α+MuIFN β[f]	6.3±1.2	4.0±2.0	-	-	128	128	-
Con-A-induced MuIFNγ[i]	32.6±1.7	-	-	35.3±2.7	-	-	-

a The quantity of MAb reacted was based on neutralization of the antiviral activity of each preparation. Since the antibody titer is determined by the inhibition of 50% of the activity (see legend of Table 1), it was necessary to add at least 2-fold more antibody to abolish all antiviral activty.

b,c,d,e Lymphokines prepared according to methods outlined in legend of Table 1.

f Pure IFNα+β was produced by Newcastle disease virus-induced mouse C-243 cells and was purchased from Enzo Biochem.

g % specific ^{51}Cr release = $\frac{\text{experimental release-spontaneous release}}{\text{total release - spontaneous release}}$ X 100

Total cell-associated ^{51}Cr determined by lysing P815 target cells with Triton X-100. Mean of triplicate samples for three separate experiments ± standard error of the mean.

h The IFN titer is defined as the reciprocal of the highest dilution of the sample which protects 50% of the assay cells (L929 B) from viral (Vesicular stomatitis virus) cytopathic effect.

i Macrophages were incubated with lymphokines for 5 hours prior to the addition of MAb.

inhibited 25-40%. In order to reduce the tumoricidal-inducing activity of all lymphokines below 50% of maximum activity, 5-10 fold more of the MAb than that needed to neutralize antiviral activity was required. At a 100-fold excess of MAb, the tumoricidal activity was reduced by 90% (results not shown). Addition of MAb to the macrophages following a 5 hr incubation with lymphokines did not inhibit tumoricidal activity. Why more MAb is required to neutralize tumoricidal activity than antiviral activity is not known, but may be related to the greater sensitivity of the tumoricidal assay.

b. Immunoadsorption of the macrophage activating factor. Anti-MAb affinity chromatography was used to demonstrate that MuIFNγ is the lymphokine which induces macrophages to become tumoricidal. The MAb was covalently coupled to Sepharose 4B beads which were then used as an immunoadsorbent to specifically bind and thus delete MuIFNγ from different preparations (Table 3). This procedure removed all the antiviral and macrophage tumoricidal activities from the different lymphokine preparations. Mock Sepharose beads failed to remove these activities from the different preparations.

These studies demonstrate that a rat MAb against MuIFNγ, initially screened for neutralization of antiviral activity, can also neutralize the ability of lymphokine preparations to activate macrophage tumoricidal activity in vitro. However, to achieve this effect, relatively more MAb was required than was needed to neutralize antiviral activity. Similar results were obtained whether unfractionated lymphokines, partially or highly purified MuIFNγ preparations were used. Published studies have shown that a MAb against human IFNγ was capable of completely inhibiting not only antiviral activity, but also the ability of these preparations from activating the secretory and microbiocidal functions of peripheral blood monocytes. The results of the combined studies with MAb's to mouse and human IFNγ, plus our findings that immobilized MAb specifically removed the factor capable of activating macrophage tumoricidal activity from lymphokine preparations, indicates that the induction of macrophage activation and expression of antiviral activity are mediated by the same molecule or by molecules sharing similar epitopes.

TABLE 3

Specific Binding of Lymphokine-induced Antiviral and Macrophage Tumoricidal Activites to Immobilized Anti-IFNγ MAb

MuIFNγ Preparation	Activities of lymphokines before and after incubation with MAb coupled to Sepharose beads [a]			
	Antiviral [g] (IFN titer)		Tumoricidal [f] (% specific ^{51}Cr Release)	
	before	after	before	after
Con-A - induced [b]	128	<4	34.6 ±1.8	1.3±1.1
PHA - induced [c]	128	<4	34.4±2.0	1.5±2.3
PHA - induced [d] (Con-A-Bound Fraction)	128	<4	33.8±1.5	1.7±1.3
PHA - induced [d] (Con-A-Unbound Fraction)	128	<4	34.4±2.0	1.5±0.7
recombinant DNA [e]	128	<4	35.0±2.1	4.9±3.1
Mock Sepharose beads without coupled MAb [a]				
PHA - induced [d]	128	128	33.2±1.5	34.1±2.2
recombinant DNA [e]	128	96	32.9±4.4	31.6±3.4

a Lymphokines were diluted to 128 IFN U/ml, mixed either with MAb coupled to Sepharose beads or mock Sepharose beads and rotated for 15 hrs. at room temp. The beads were then pelleted at 200xg for 5 min. and the supernatant tested for antiviral and tumoricidal-inducing activities. Mock beads were generated by treating CNBr-activated Sepharose-4B, in the absence of protein, under identical conditions used for the coupling of MAb.

b,c,d,e Lymphokines perpared according to methods outlined in legend of Table 1.

f See legend of Table 2 for description of tumoricidal assay.

g See legend of Table 2 for definition of IFN titer.